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## Antihypoxic potentiation of standard therapy for experimental colorectal liver metastasis through myo-inositol trispyrophosphate

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**Abstract:** **PURPOSE:** Tumor hypoxia activates hypoxia-inducible factors (Hifs), which induce a range of malignant changes including vascular abnormalities. Here, we determine whether inhibition of the hypoxic tumor response through myo-inositol trispyrophosphate (ITPP), a compound with antihypoxic properties, is able to cause prolonged vascular normalization that can be exploited to improve standard-of-care treatment. **EXPERIMENTAL DESIGN:** We tested ITPP on two syngeneic orthotopic mouse models of lethal colorectal cancer liver metastasis. Tumors were monitored by MRI and analyzed for the hypoxic response and their malignant potential. A Hif activator and in vitro assays were used to define the working mode of ITPP. Hypoxic response and vasculature were re-evaluated 4 weeks after treatment. Finally, we determined survival following ITPP monotherapy, FOLFOX monotherapy, FOLFOX plus Vegf antibody, and FOLFOX plus ITPP, both overlapping and sequential. **RESULTS:** ITPP reduced tumor load, efficiently inhibited the hypoxic response, and improved survival. These effects were lost when mice were pretreated with a Hif activator. Its immediate effects on the hypoxic response, including an apparent normalization of tumor vasculature, persisted for at least 4 weeks after treatment cessation. Compared with FOLFOX alone, Vegf antibody combined with FOLFOX prolonged survival by <30%, whereas ITPP combined with FOLFOX extended survival by >140%, regardless of whether FOLFOX was given in overlap or after ITPP exposure. **CONCLUSIONS:** Our findings reveal a truly antihypoxic mechanism for ITPP and demonstrate the capacity of this nontoxic compound to potentiate the efficacy of existing anticancer treatment in a way amenable to clinical translation. Clin Cancer Res; 22(23); 5887-97.

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# **Antihypoxic Potentiation of Standard Therapy for Experimental Colorectal Liver Metastasis through Myo-Inositol Trispyrophosphate**

**Authors:** Perparim Limani<sup>1</sup>, Michael Linecker<sup>1</sup>, Ekaterina Kachaylo<sup>1</sup>, Christoph Tschuor<sup>1</sup>, Philipp Kron<sup>1</sup>, Andrea Schlegel<sup>1</sup>, Udo Ungethuem<sup>1</sup>, Jae Hwi Jang<sup>1</sup>, Stavroula Georgiopoulou<sup>2</sup>, Claude Nicolau<sup>3</sup>, Jean-Marie Lehn<sup>4</sup>, Rolf Graf<sup>1</sup>, Bostjan Humar<sup>1\*</sup>, Pierre-Alain Clavien<sup>1\*</sup>

\*shared senior authorship

**Affiliations:** <sup>1</sup>Department of Surgery, Swiss Hepato-Pancreato-Biliary (HPB) and Transplantation Center, University Hospital Zurich, Raemistrasse 100, Zurich, CH-8091, Switzerland. <sup>2</sup>Department of Internal Medicine, Research Unit, University Hospital Zurich, Raemistrasse 100, Zurich, CH-8091, Switzerland. <sup>3</sup>Friedman School of Nutrition Science and Policy, Tufts University, 150 Harrison Ave, Boston, MA 02111, USA. <sup>4</sup>Institut de Science et d'Ingénierie Supramoléculaires (ISIS), Université de Strasbourg, 8 Allée Gaspard Monge, Strasbourg, F-67000, France

**Running title:** Chemotherapy potentiation through antihypoxic ITTP

**Key words:** myo-inositol trispyrophosphate, tumor hypoxia, vascular normalization, potentiation, standard chemotherapy.

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**Correspondence:** Pierre-Alain Clavien, Department of Surgery, University Hospital Zurich, Raemistrasse 100, CH-8091 Zurich, Switzerland, Phone: +41-44-255 23 00, E-mail: clavien@access.uzh.ch

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**Word count:** 4149. **Number of figures:** 6.

### **Translational relevance**

Besides the promotion of malignant behavior, tumor hypoxia induces brittle vasculature that impedes the efficient delivery of chemotherapeutic agents. Inositol trispyrophosphate (ITPP) is a novel antihypoxic compound known to inhibit the hypoxic tumor response and to improve drug delivery if given concomitantly. More so, ITPP lacks significant toxicity and hence may effectively be used in the clinic for the improvement of existing treatments. Here, we provide important, but currently lacking data to inform future phase II/III trials on ITPP. We illustrate the main working mode of ITPP, show that the antihypoxic/vascular effects of ITPP are sustained, and demonstrate the superiority of ITPP in potentiating subsequent chemotherapy effects. Acting as a direct inhibitor of tumor hypoxia, ITPP treatment generates a time window that can be exploited to enhance existing chemotherapy regimens. A phase Ib/IIa trial is currently assessing the safety of ITPP in cancer patients.

## Abstract

**Purpose.** Tumor hypoxia activates hypoxia-inducible factors (Hifs), which induce a range of malignant changes including vascular abnormalities. Here we determine whether inhibition of the hypoxic tumor response through myo-inositol trispyrophosphate (ITPP), a compound with antihypoxic properties, is able to cause prolonged vascular normalization that can be exploited to improve standard of care treatment.

**Experimental design.** We tested ITPP on two syngeneic orthotopic mouse models of lethal colorectal (CRC) liver metastasis. Tumors were monitored by MRI and analyzed for the hypoxic response and their malignant potential. A Hif activator and *in vitro* assays were used to define the working mode of ITPP. Hypoxic response and vasculature were re-evaluated four weeks after treatment. Finally, we determined survival following ITPP monotherapy, FOLFOX monotherapy, FOLFOX plus Vegf antibody, and FOLFOX plus ITPP, both overlapping and sequential.

**Results.** ITPP reduced tumor load, efficiently inhibited the hypoxic response, and improved survival. These effects were lost when mice were pretreated with a Hif activator. Its immediate effects on the hypoxic response, including an apparent normalization of tumor vasculature, persisted for at least four weeks after treatment cessation. Compared to FOLFOX alone, Vegf antibody combined with FOLFOX prolonged survival by <30%, whereas ITPP combined with FOLFOX extended survival by >140%, regardless of whether FOLFOX was given in overlap or after ITPP exposure.

**Conclusions.** Our findings reveal a truly antihypoxic mechanism for ITPP and demonstrate the capacity of this nontoxic compound to potentiate the efficacy of existing anticancer treatment in a way amenable to clinical translation.

## Introduction

Tumor hypoxia activates the hypoxia-inducible factors (Hifs), which induce an adaptive response associated with an array of processes that foster tumor progression (1-4). Most famous is the angiogenic switch, leading to the formation of immature, leaky vessels that however cannot re-install normoxia but instead enhance the spread of disease (5). Vascular normalization - the reversal to a more physiological network of mature and tight vessels - is a goal aspired by modern therapies, particularly because it also promotes the delivery and efficacy of anticancer compounds (5). However, hypoxia drives many other processes, including changes in energy metabolism (Warburg effect), chronic inflammation, suppression of adaptive immunity, invasive behavior, or an increased tumorigenic potential (6-10). Considering this complexity, the arguably simplest approach to normalize vessels and combat malignant behavior is the targeting of hypoxia *per se*.

Myo-inositol trispyrophosphate (ITPP) is a novel molecule that combines the necessary features for the successful treatment of tumor hypoxia (11). Acting as an allosteric effector of hemoglobin, ITPP increases dissociation of O<sub>2</sub> from heme particularly under low pO<sub>2</sub>, creating antihypoxic potency (11). Additionally, its uptake seems to be tissue-specific towards erythrocytes (12). More so, ITPP does display little, if any, *in vivo* toxicity, neither in animals (13-16) nor humans (<http://normoxys.com/clinical-trial-results/>). In preclinical studies, ITPP markedly improved outcomes in hepatoma, colorectal cancer (CRC), breast cancer, melanoma, and pancreatic cancer (13-16). Combined evidence from these studies indicates ITPP restores tumor normoxia, downregulates Hif/Vegf, and inhibits processes associated with the hypoxic response. Notably, clear signs of vascular normalization were likewise reported. Owing to the improvements in vessel structure, ITPP strongly potentiated the effects of gemcitabine in a syngeneic model of pancreatic cancer (15, 16).

Despite these impressive effects, several questions remain open before exploring ITPP in human cancer. Although the working mode of ITPP seemingly relies on the regain of tumor

normoxia and the subsequent stalling of the hypoxic response, a formal prove for this is lacking and direct effects on cancer cells remain possible (15, 16). The potentiation of cytotoxic chemotherapy through ITPP was shown in the setting of concomitant application (15, 16). In clinical practice however, sequential (chemotherapy follows ITPP treatment) rather than concomitant application would be more feasible both in terms of ethical concerns and toxicity control. Likewise, how the combination of ITPP with chemotherapy will perform against current combination approaches that consider vascular normalization (i.e. anti-angiogenic therapy) is unknown.

Here, we tested ITPP on two syngeneic orthotopic mouse models of CRC liver metastasis, the most frequent cause of death due to CRC (17). Our experiments were designed to i) clarify the working mode of ITPP, to ii) establish a time window associated with vascular normalization through ITPP, and to iii) estimate the clinical efficacy of ITPP-enhanced chemotherapy by comparing with the current standard FOLFOX plus neutralizing Vegf antibody.

## **Methods**

### **Animals**

All animal experiments were in accord with Swiss Federal Animal Regulations and approved by the Veterinary Office of Zurich. Male mice (C57Bl/6 and BALB/c, from Harlan. Itingen, Switzerland) aged 10-12 weeks were kept on a 12-hour day/night cycle with free access to food and water.

### **Animal Procedures, Treatment, and MRI Imaging**

Selective portal vein injection was used for the generation of CRC liver metastasis, with concurrent clamping of distal liver lobes leading to tumor development restricted to the right and caudate lobes. Thereby, survival mainly depends on the cancer development within affected lobes (manuscript under revision). ITPP was synthesized as previously described (11) and administered as a solution containing calcium chloride to balance its chelating effects (13). ITPP was injected i.v. five times every 48h in a volume of 100 µl at 500mg/kg (Fig. 1A). Controls received saline. DMOG (BML-EI347-0050, Enzo Life Science, Farmingdale, New York; 40 mg/kg in 100 µl saline) was i.v. injected 4h before every ITPP injection (18). FOLFOX (6 mg/kg oxaliplatin, followed 2h later by 90 mg/kg folinic acid and 50 mg/kg 5-fluorouracil, Sigma-Aldrich, Dorset, UK) was given 5x every other day either alone for monotherapy (akin to ITPP, Fig. 1A), or 24h following ITPP administration (overlapping mode) or 24h after the last ITPP dose (sequential mode) for combination therapy (19). Controls received saline. Anti-Vegf antibody or control IgG (4 µg/kg, R&D Systems, Abingdon UK) were injected i.v. concomitant with FOLFOX as described (20). MRI was performed on a Bruker Biospin 4.7 Tesla imager (Ettlingen, Germany) at the day 7, 17, 45, or at time of death in the survival studies.

### **Blood oxygen saturation**

Oxygen dissociation curves (P50 values) were determined with a HEMOX Analyzer (TCS Scientific, New Hope, PA). The percentage of O<sub>2</sub> hemoglobin saturation was continuously

recorded in 50  $\mu$ L blood mixed with 3 mL Hemox buffer and 10  $\mu$ L anti-foaming agent equilibrated at 37°C. Chart software (AD Instruments, Colorado Springs, USA) was used for analysis (13).

### **Cell culture and *ex vivo* experiments**

Syngeneic CRC cells MC-38 (C57Bl/6 background, a kind gift from Carole Bourquin) and CT-26 (BALB/c background, LGC Promochem, Molsheim, France) were cultured as reported (21). Freshly resected tumors were sequentially minced through a 100 $\mu$ m and a 30 $\mu$ m mesh (BD Falcon<sup>TM</sup>, Franklin Lakes, NJ) into single cells. Invasion assays were performed using 8 $\mu$ m pore cell culture inserts and Matrigel (BD Falcon<sup>TM</sup>) as described (22). For s.c. injections, 100  $\mu$ L of single cell preparation from resected tumors at  $5 \times 10^6$  cells/ml in PBS were used. Glucose and lactate levels were determined with a Fuji Dri-Chemi 4000i (Fujifilm Corporation, Tokyo, Japan). All *in vitro* experiments were performed in triplicates.

### **Histological assessment**

Hematoxylin-Eosine staining was performed on 3 $\mu$ m archived liver sections. For immunohistochemistry, antigens were retrieved by boiling in citrate buffer. Secondary detection was done using the Ventana Discovery automated staining system and the iView DAB kit (Ventana Medical Systems, Tucson, AZ). See Supplements for primary antibodies.

### **Real-Time Polymerase Chain Reaction, Western blots, ELISA**

qRT-PCR and immunoblots were performed as described (20). See Supplements for further information.

### **Statistical Analysis**

At least six animals/group were analyzed unless specified otherwise. Differences between the groups were assessed by a 2-tailed t test assuming unequal variance, or by one-way analysis



of variance where applicable. Statistical significance was set at  $p < 0.05$ , with data presented as means and 95% confidence intervals. Prism 5.0 (GraphPad, San Diego, CA) software was used.

## **Results**

### **Impact of ITPP on tumor development**

The impact of ITPP was tested on two syngeneic orthotopic models of CRC liver metastasis (MC-38 and CT-26 CRC cells injected into the portal vein of C57BL/6 and BALB/c mice, respectively). These models feature tumors restricted to the right and caudate liver lobes, with survival largely dependent on the intralobular tumor development (manuscript under revision).

ITPP treatment (Supplementary Fig. 1A) was started after tumor diagnosis by MRI at day 7 and was repeated five times every other day (Fig. 1A). After treatment (day 17), tumors were harvested and analyzed (Fig. 1B). Compared to saline-treated tumors, ITPP-treated tumors displayed reduced proliferative activity, increased apoptosis, and decreased DAMP levels. Likewise, ITPP treatment reduced both the number and volume of tumors per animal (Fig. 1B, Supplementary Fig. 2A).

### **Impact of ITPP on tumor hypoxia and animal survival**

The impact of ITPP on tumor hypoxia was assessed through Hif expression and pimonidazole staining. In both tumor models, ITPP led to nuclear downregulation of Hif1a and Hif2a compared to controls. Likewise, pimonidazole staining indicated a strong reduction in hypoxic tumor areas through ITPP (Fig. 1C, Supplementary Fig. 2B). Since hypoxia/HIF1a can predict outcome in metastatic CRC (2-4), we examined survival of tumor-bearing mice. ITPP prolonged survival by 50% (MC-38/B6 model, Fig. 1D, median survival ( $\pm 95\%$ CI) from treatment end:  $37.8 \pm 2.09$  days for controls and  $56.7 \pm 3.61$  days for ITPP-treated animals) and 45% (CT-26/BALB model, Supplementary Fig. 2C, survival  $37.1 \pm 2.42$  days for controls and  $53.9 \pm 2.54$  days for ITPP-treated animals) relative to controls, with animals dying from portal congestion due to tumor growth. Therefore, the anti-hypoxic and growth inhibitory actions of ITPP are associated with an improved outcome.

### **Impact of ITPP on the hypoxic tumor response**

To demonstrate inhibition of the hypoxic response by ITPP, we assessed a series of markers related to hypoxia-driven processes. Overall, ITPP-treatment was associated with the inhibition of the Warburg effect (a shift from glycolysis to  $\beta$ -oxidation, Fig. 2A, Supplementary Fig. 3A), with reduced inflammation, and the increased influx of immune cells (Fig. 2B, Supplementary Fig. 3B). Increased macrophage infiltration was associated with M1 polarization marker expression, consistent with an anticancerous phenotype (23). Additionally, we observed in ITPP-treated relative to control tumors downregulation of markers of the epithelial-mesenchymal transition (EMT) (9) along with congruent changes in cancer cell morphology (Fig. 3A/B, Supplementary Fig. 4A/B), and in sera from ITPP-treated mice a relative reduction in tumor-independent, systemic promoters of malignancy (Fig. 3C, Supplementary Fig. 4B), which typically are induced by hypoxia-associated inflammation (24).

To substantiate the novel finding of a reduced malignant potential following ITPP treatment, we isolated on day 17 cancer cells from our MC-38/B6 model and assessed their *ex vivo* invasiveness. Relative to cells from control tumors, the invasion of ITPP-exposed cancer cells through Matrigel was reduced by >50% (Fig. 3D). Moreover, when *ex vivo* cancer cells were s.c. injected into recipient mice, cells from control tumors formed nearly 3x larger tumors than cells from ITPP tumors (Fig. 3E), indicating that ITPP reduces both invasiveness and tumorigenic potential. Together, these findings indicate ITPP efficiently inhibits the hypoxic response in experimental liver metastasis.

### **Dependency of ITPP action on hypoxia-inducible factors**

ITPP may inhibit the hypoxic response via Hif downregulation. If so, exogenous activation of Hifs should counteract its effects. Tumor-bearing mice (MC-38/B6) were injected with ITPP or saline and co-treated or not with the generic Hif activator dimethyloxaloylglycine (DMOG) (25). In both saline and ITPP treated tumors, DMOG promoted all parameters positively related to the hypoxic response. Vice versa, ITPP antagonized these changes in vehicle- or DMOG-exposed tumors. Notably, ITPP effects appeared to be neutralized by the addition of DMOG

(Fig. 4A-C). Indeed, while survival was prolonged in the ITPP group ( $65.5 \pm 2.79$  days versus  $35.5 \pm 3.03$  for controls), survival in the ITPP plus DMOG group ( $41.5 \pm 2.98$ ) was similar to saline controls. The DMOG group ( $27.5 \pm 2.81$ ) tended to have the worst outcome (Fig. 4D). We conclude that the inhibition of Hifs is a main action mechanism underlying the anti-cancerous properties of ITPP.

### **Direct effects of hypoxia and ITPP on cancer cells**

ITPP might modulate Hifs via hypoxia-independent mechanisms. To clarify, we exposed hypoxic MC-38 and CT-26 cultures to normoxia and examined aspects of the hypoxic response. Similar to ITPP *in vivo*, normoxia *in vitro* reduced growth, counteracted the Warburg effect (increased mitochondrial, reduced glycolytic activity), and inhibited invasiveness of cancer cells (Supplementary Fig. 5A-C). In contrast, the addition of ITPP to hypoxic cultures had no impact on investigated parameters (Supplementary Fig. 5D-F). Therefore, the ITPP effects on tumors are indirect, can be mimicked through normoxia, are dependent on Hif inhibition, and consequently must be mediated by the inhibition of hypoxia.

### **Impact of ITPP on tumor-associated vascular normalization**

ITPP downregulates angiogenic molecules, including Vegf and Plgf (5, 24, 26) (Fig. 4C, Supplementary Fig. 4B). On histology, the density of Cd31/34-positive vessels was visibly reduced in ITPP-treated versus control tumors. Vice versa, the density of vessels positive for Notch1 and VE-cadherin, both central for the maintenance of a mature and tight vessel phenotype (27-31), was increased in ITPP-treated tumors (Supplementary Figs. 6 & 7). Therefore, ITPP treatment of tumors is associated with vascular normalization.

### **Long-term impact of ITPP on the hypoxic tumor response**

The observed endothelial changes are consistent with a functional vascular normalization through ITPP, as has been reported for other preclinical models (15, 16). If ITPP-induced vessel normalization is functional also in our CRC metastasis model, suppression of the hypoxic

response should be long-lasting. We analyzed tissue/serum from tumor-laden mice at four weeks after treatment (day 45, before controls start to die) and compared with the time immediately after treatment (day 17). The hypoxic response was assessed through *Hif1a*, *Hif2a* & *Vegfa* and markers related to glycolysis (*Slc2a*, *Ldha*), mitochondrial mass (*Cox4i1*),  $\beta$ -oxidation (*Cpt1a*, *Hadha*, *Hadhb*), mitochondrial dynamics (*Fis1*, *Drp1*, *Mfn2*, *Opal*), inflammation (*Tnfa*, *Il6*, *Emr1*), macrophage polarization (*Cd40*, *Nos2a*, *Arg1*, *Mrc1*, *Mgl1*), the EMT (*Twist1*, *Snail*, *Src*, *Vim*, *Ocln*, *Cdh1*), angiogenesis (Vegf, Plgf), necrosis/inflammation (Hmgb1), and systemic tumor promotion (Scf, osteopontin, Cxcl12). Comparison of time points revealed ITPP treatment induces similar qualitative and quantitative changes at day 17 and 45 (Fig. 5A, Supplementary Fig. 8A), indicating a long-range stalling of the hypoxic response through ITPP. Re-examination of endothelial proteins indicated the persistence of a reduced Cd31/34-microvessel density along with an increase in mature vessels at four weeks after ITPP treatment (Fig. 5B, Supplementary Fig. 8B). We conclude that ITPP induces a stable, long-lasting tumor response which is accompanied by vascular normalization.

### **Impact of ITPP on cytotoxic chemotherapy in comparison to anti-angiogenic therapy**

The ITPP effects on tumor-associated vasculature are expected to improve the delivery and efficacy of cytotoxic regimens (16). The FOLFOX regimen is a standard for metastatic CRC, and nowadays often is combined with antiangiogenic VEGF antibody (32-34). To determine whether ITPP may improve the effects of FOLFOX, we treated tumor-bearing mice (MC-38/B6) with ITPP, the drugs of the FOLFOX regimen (here referred to as FOLFOX), or a combination thereof. To estimate the potential clinical efficacy, we compared to FOLFOX plus neutralizing anti-mouse Vegf antibody (Fig. 6A). Compared to controls (median survival from treatment end =  $35.5 \pm 3.03$  [days $\pm$ 95%CI]), FOLFOX extended survival by 75% [ $62 \pm 3.03$ ], akin to ITPP monotherapy [ $65.5 \pm 2.79$ ](Fig. 8A). When combined with Vegf antibody, FOLFOX prolonged survival by 225% [ $80 \pm 2.55$ ]. However, when combined with ITPP (overlapping mode), survival was extended by 431% [ $153 \pm 3.34$ ], indicating synergistic efficacy ( $p < 0.001$  when comparing additive effects of FOLFOX and ITPP monotherapy with combination

treatment). Importantly, a similar (428%) survival advantage [ $152 \pm 4.17$ ] was achieved when FOLFOX was given after ITPP treatment (sequential mode,  $p < 0.001$  for comparing additive effects with combination), consistent with a lasting effect of ITPP on vasculature.

When assessing tumor load, only small differences were noted between the different treatments regarding tumor volume (Fig. 6B/C). ITPP alone or with FOLFOX however had the strongest impact on tumor multiplicity, an effect that persisted until death. Altogether, these findings demonstrate a marked potency of ITPP to enhance classic chemotherapy well beyond the efficacy of standard-of-care treatments.

## Discussion

In this study, we demonstrate that the effects of ITPP on experimental liver metastases rely on the inhibition of the hypoxic response via Hif downregulation secondary to the restoration of tumor normoxia. Furthermore, we show that the inhibition of the hypoxic response is sustained and associated with vascular normalization, providing a generous window of therapeutic opportunity. Consequent to this lasting response, ITPP potentiates cytotoxic chemotherapy given in overlapping or sequential mode, pointing to the functionality of prolonged vascular normalization and resulting in a marked survival advantage when compared to chemotherapy alone or combined with an antiangiogenic agent.

In keeping with previous studies (13-16), ITPP treatment of mice carrying syngeneic orthotopic CRC liver metastases reduced tumor load, inhibited hypoxia, Hifs, the hypoxic response, promoted vessel normalization, and improved survival. Novel findings include the increased influx of macrophages with M1 polarization expression profiles, a change that may result from the ITPP-associated drop in Plgf (35) and may directly promote vessel normalization (36). Furthermore, we provide functional evidence for the inhibition of a malignant phenotype through ITPP, in terms of both invasiveness and colony-forming capacity. A general reduction in malignant potential was underscored by the downregulation of tumor-independent metastatic promoters, consistent with a systemic action of ITPP.

The ability of ITPP to suppress the hypoxic response is the upshot of improved tumor oxygenation leading to Hif downregulation. Exogenous activation of Hifs via DMOG promoted exemplary facets of the hypoxic response in the absence or presence of ITPP, indicating Hif activity, not oxygenation as such, drives the hypoxic response. ITPP lost its impact on the hypoxic response and survival in the presence of DMOG, had no impact on cancer cells *in vitro*, whereas *in vitro* normoxia in cancer cells mimicked its *in vivo* effects. Therefore, the main action mechanism of ITPP is the provision of oxygen followed by Hif downregulation, whereas direct effects on cancer cells seem negligible.

The potent anti-hypoxic capacities of ITPP are associated with a remarkable extension of its effects, lasting at least a month post treatment. Vascular normalization hence is stable and provides opportunity for improving standard therapy. Compared to FOLFOX alone, the addition of ITPP prolonged survival by 2.45x, regardless whether given in overlapping or sequential mode. Therefore, ITPP enables functional vessel normalization that can be exploited following ITPP cessation. This finding provides a strong argument for the integration of ITPP into existing therapeutic protocols, because a sequential application (first ITPP, then chemotherapy) facilitates trial design and minimizes the risk of potential, harmful drug interactions. The estimation of clinical efficacy from animal studies remains difficult; the comparison of ITPP-FOLFOX to the standard-of-care FOLFOX-Vegf antibody however suggests ITPP has a strong potential to improve clinical outcomes, albeit with the limitation that FOLFOX administration in mice was different from that in humans. Vegf antibody modestly enhanced FOLFOX efficacy (1.29x), comparable to what is known from clinical studies (32-34). Therefore, our experimental set up seems a clinically valid estimate of treatment effects. Under given conditions, FOLFOX-ITPP clearly outperformed FOLFOX-Vegf antibody treatment. Our experiments were not designed to mechanistically explain the superiority of ITPP over Vegf inhibition. However, antiangiogenic therapy has its conceptual limitations (i.e. enhancement of hypoxia, targeting of only one aspect of the hypoxic response) and is suspected to increase metastatic risk (37, 38), perhaps explaining its small impact on overall survival (5, 32-34). By inhibiting the hypoxic response through normoxic restoration, ITPP should be largely devoid of such risks.

A potential danger however may be ITPP administration at too wide intervals. Such application could lead to fluctuations in tissue O<sub>2</sub>-levels as is known from intermittent hypoxia, thought to be an even stronger driver of malignancy than chronic hypoxia (39). Notably, sufficient provision of ITPP over longer periods seems feasible. Given that ITPP acts in an untargeted, systemic way, the risk of resistance development likely is small compared to targeted agents (40, 41). More importantly, ITPP has not been associated with toxicity thus far. In all animals studies including ours, ITPP was very well tolerated (13-15, 42), and no impact



on hematological parameters has been reported (13). Minor *in vitro* toxicity was noted, however only at doses ~30x higher than we used (16). A phase Ia trial on healthy human volunteers (n=36) likewise concluded with an absence of drug-related adverse effects (<http://normoxys.com/clinical-trial-results/>). Indeed, ITPP even may improve the physical performance status (42), a relevant issue for sick and often elderly cancer patients. Side effects associated with antiangiogenic therapy are clearly not expected (32, 34), but how ITPP will be tolerated in cancer patients is unknown. A phase Ib/IIa clinical trial (NCT02528526) is currently addressing dosing in patients with gastrointestinal malignancy.

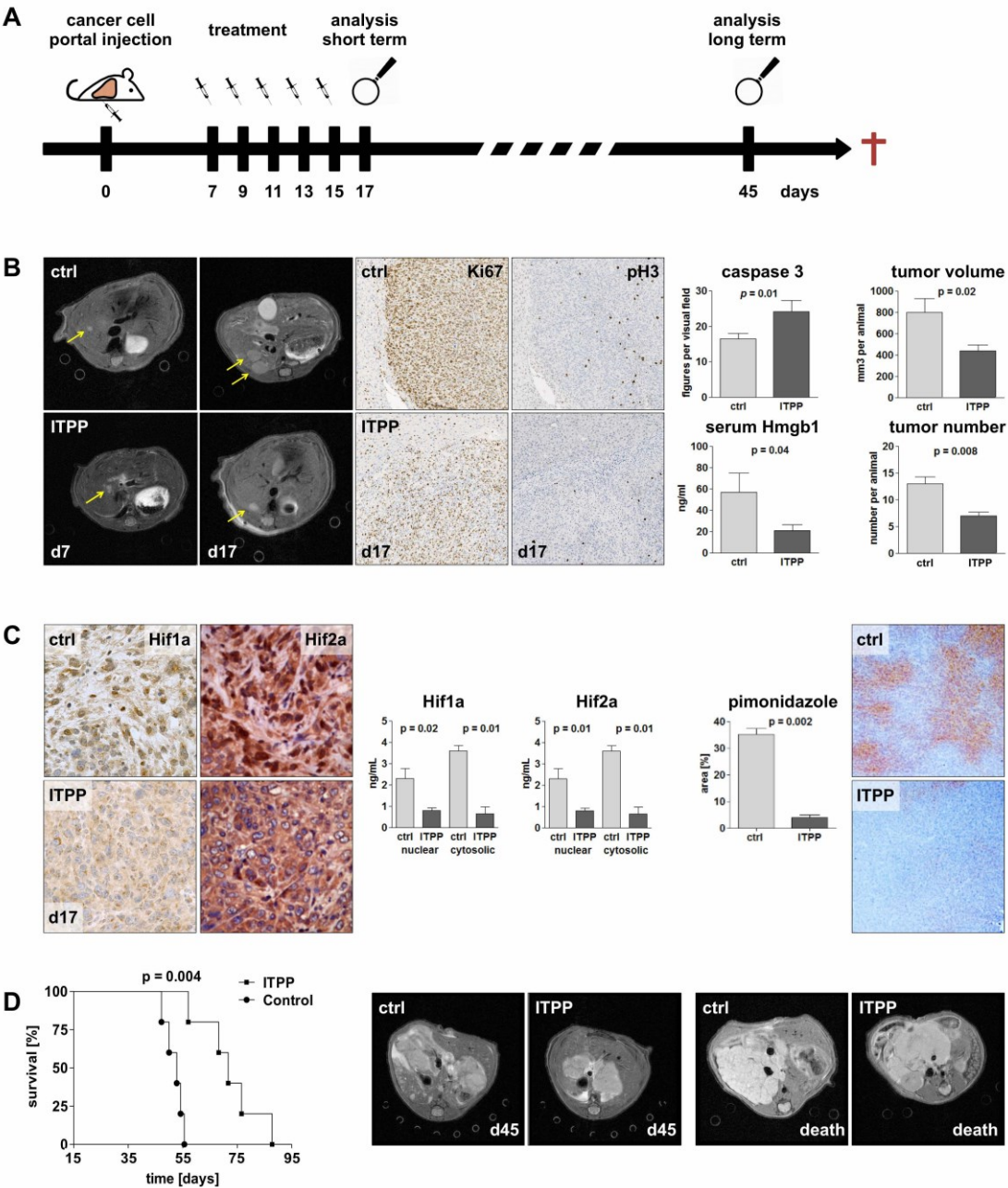
In summary, we provide key information required to translate the remarkable anticancer effects of ITPP into the clinic. We show that ITPP efficiently inhibits various aspects associated with the hypoxic tumor response, including the malignant behavior of cancer cells. We demonstrate that the ITPP effects rely on the restoration of normoxia and the subsequent downregulation of Hifs, offering an action mechanism and classifying ITPP as a true antihypoxic compound. We further show that vascular normalization achieved through ITPP is stable and paralleled by a long-lasting inhibition of the hypoxic response, providing ample opportunity to combine with and boost the effects of current standard chemotherapy. Finally, we observed in a one-to-one comparison the superior performance of ITPP against neutralizing Vegf antibody when combined with cytotoxic regimens. Together, these findings call for a rapid clinical evaluation of the nontoxic, antihypoxic ITPP as a novel cancer treatment.

## **Acknowledgements**

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# Figure Legends

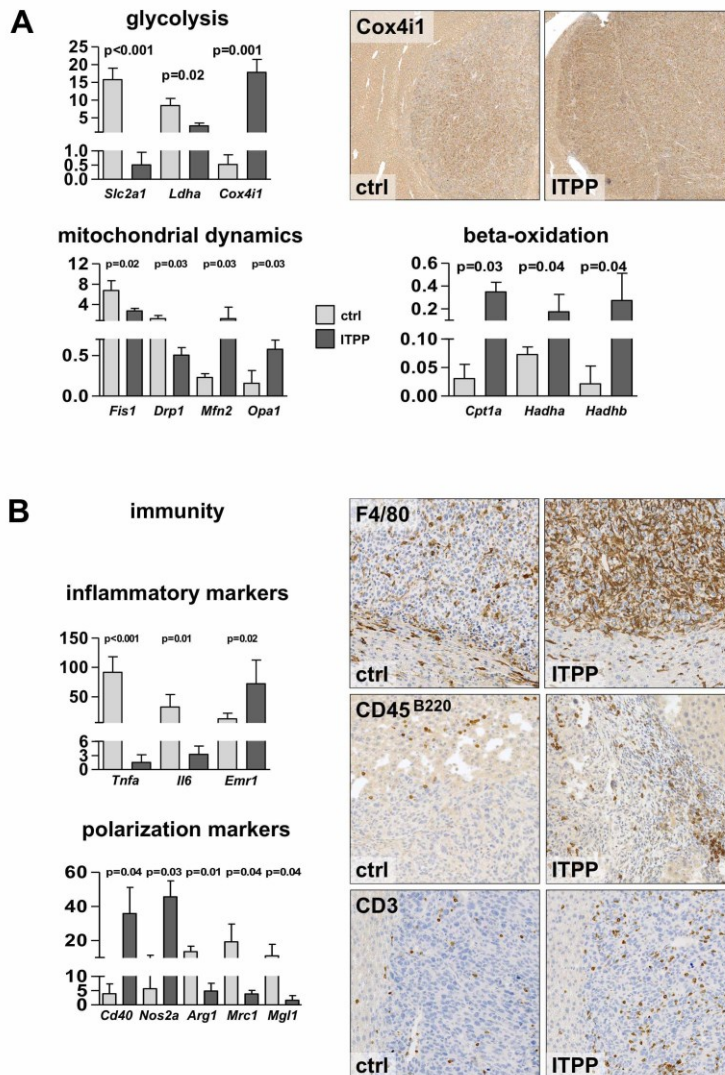
**Figure 1**



**Figure 1.** Impact of ITPP on tumor burden, hypoxia and survival in the MC-38/B6 model. A, General treatment schedule for ITPP. C57Bl/6 mice were portally injected with the same number of MC-38 cancer cells and randomly assigned to control or treatment groups upon MRI diagnosis on day 7. B, Tumor response towards ITPP. Analysis included tumor development (representative MRI scans before and after treatment, ctrl=saline control), proliferation (Ki67,

pH3, magnification 10x), apoptosis (caspase 3), necrosis/inflammation (serum Hmgb1), and tumor volume/number (pathological and MRI assessment) after treatment. C, Impact on tumoral Hif expression and hypoxia. Nuclear Hif1a and Hif2a expression (magnification 40x), nuclear and cytoplasmic Hif levels (ELISA), and pimonidazole staining for hypoxic areas (magnification 5x) immediately after treatment are shown. D, Kaplan-Meier survival analysis. Day 15 represents treatment end (n=5/group). Representative MRI scans show tumor load at day 45 (28 days after treatment) and at the time of death, associating death with the preoccupation of the right and caudate liver lobe.

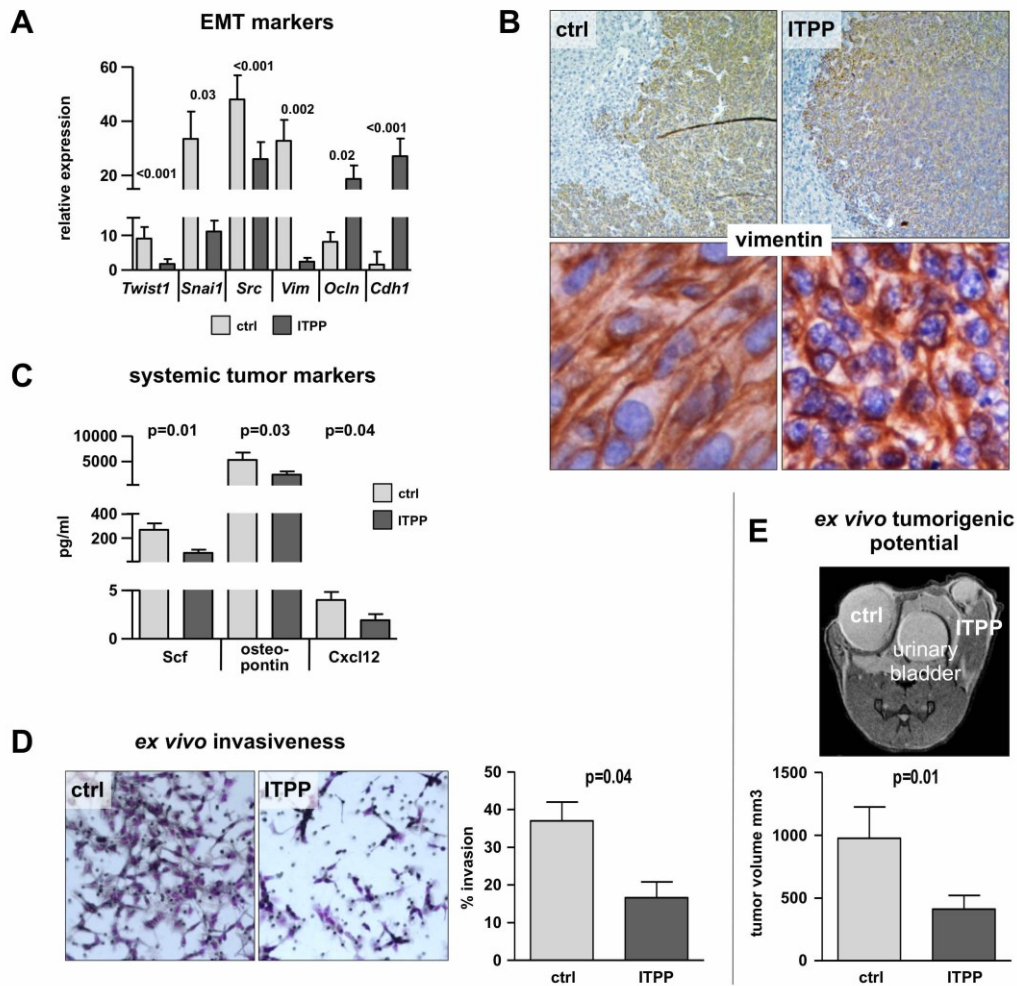
**Figure 2**



**Figure 2.** Impact of ITPP on metabolic and immune parameters in the MC-38/B6 model at day 17. A, Impact on energy metabolism. Graphs show normalized gene expression in control tumors (light gray bars) and ITPP-treated tumors (dark gray bars) for glycolysis (glucose transporter *Slc2a1*, lactate dehydrogenase *Ldha*, mitochondrial marker *Cox4i1*), mitochondrial dynamics (mitochondrial fission *Fis1* & *Dsp1*, mitochondrial fusion *Mfn2* & *Opa1*), and  $\beta$ -oxidation genes (*Cpt1a*, *Hadha*, *Hadhb*). Images (top right) show immunostaining for Cox4i1 protein in control (ctrl) and ITPP-treated tumors (magnification 10x). B, Impact on immunity. Graphs to the left show normalized expression of inflammatory (upper graph; *Tnfa*, *Il6*, and *Emr1* encoding the macrophage marker F4/80) and macrophage polarization markers (lower

graph; M1 markers: *Cd40*, *Nos2a*; M2 markers: *Arg1*, *Mrc1*, *Mgl1*). Images to the right (magnification 20x) show increased tumor infiltration of ITPP-treated tumors by macrophages (F4/80), B-cells (CD45<sup>B220</sup>), and NK cells (CD3).

**Figure 3**

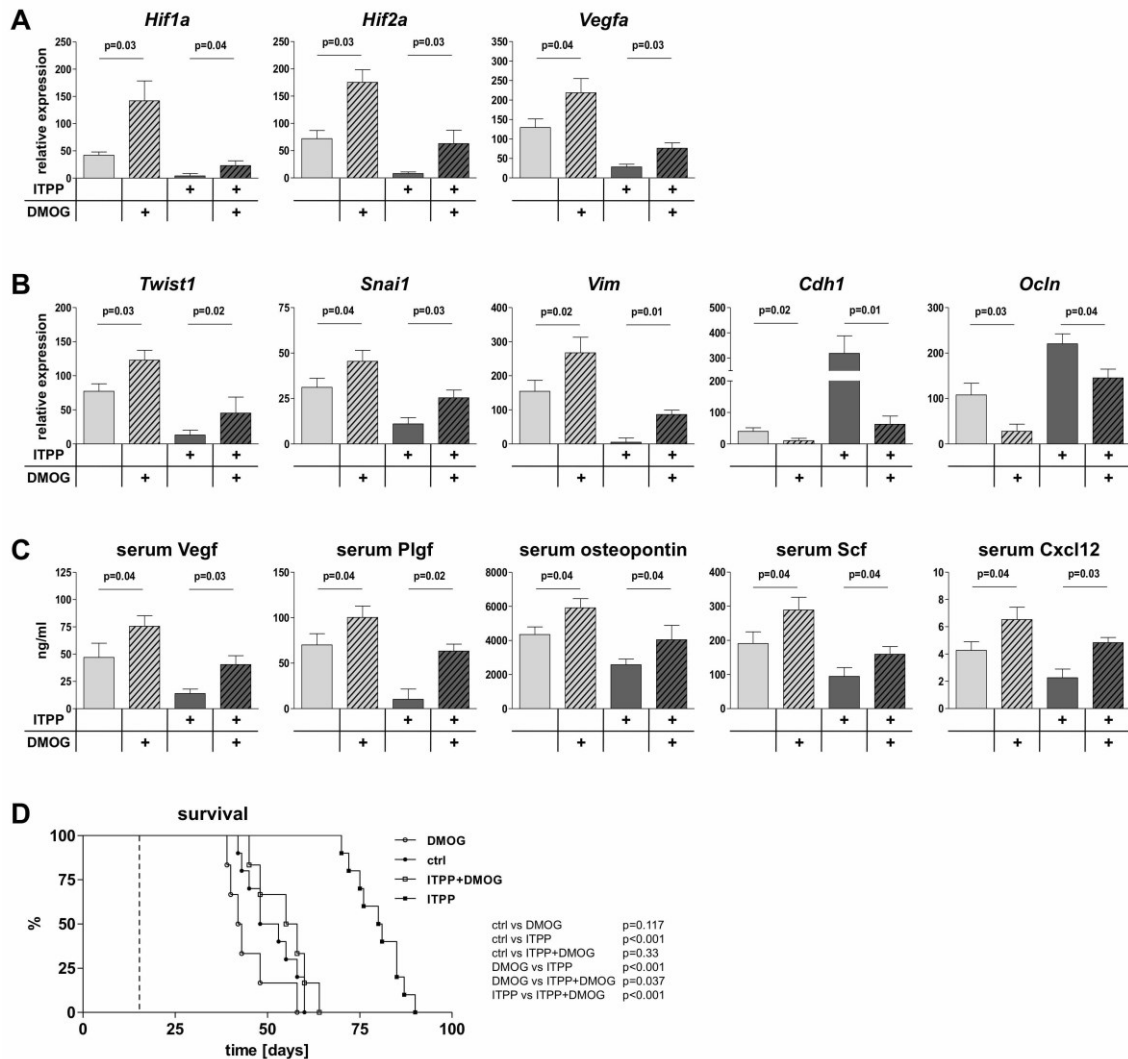


**Figure 3.** Impact of ITPP on malignant tumor phenotype in the MC-38/B6 model at day 17. A, Normalized gene expression of EMT inducers (*Twist*, *Snai1*, *Src*), a mesenchymal marker (*Vim*) and epithelial markers (*Ocln*, *Cdh1*) in control and ITPP tumors. B, Immunohistochemistry for vimentin (magnification 10x/100x for upper/lower panels) in control and ITPP-treated tumors. Note the fibroblastoid cancer cell morphology in control tumors, and the more condensed, spheroid morphology in ITPP-treated tumors. C, Circulating levels of tumor-independent, systemic promoters of malignancy (Scf, osteopontin, Cxcl12) in control and ITPP-treated tumors. D, *Ex vivo* invasion assay on cancer cells freshly isolated from tumors immediately after treatment. Images show cancer cells derived from control or ITPP-treated tumors that have invaded through Matrigel (Boyden-chamber-assay). Invasiveness was quantified (% of cells that have invaded through the insert) for one tumor from each mouse (n=6/group). E, *Ex vivo* assay

for tumorigenic potential. The MRI scan shows tumor development at day 14 after s.c. injection of cancer cells derived from control or ITPP-treated tumors. The object in the middle represents the bladder. Quantification is based on the mean tumor volume from MRI scans. Cell suspensions were prepared from tumors of 6 mice/group and injected into 6 mice.



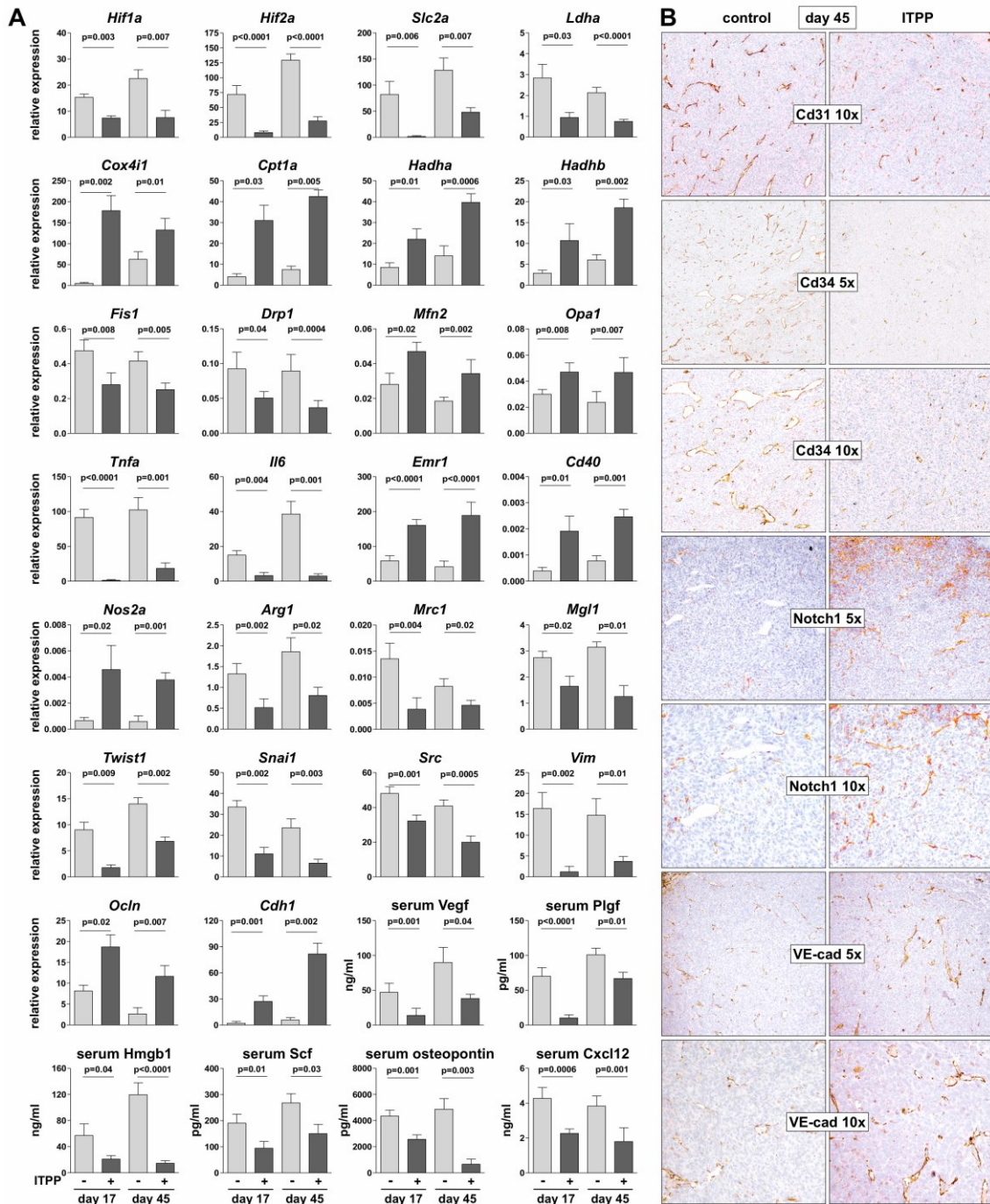
**Figure 4**



**Figure 4.** Impact of exogenous Hif activation on the hypoxic response and on ITPP effects. The Hif activator DMOG was i.v. injected 4h prior to each ITPP or saline application into B6 mice carrying MC-38 tumors. After treatment (day 17), hepatic gene expression and serum markers were analyzed. A, Hif analysis. Normalized gene (*Hif1a*, *Hif2a*, *Vegfa*) expression in tumors exposed to DMOG vehicle and saline, to DMOG and saline, to DMOG vehicle and ITPP, and to DMOG and ITPP. B, EMT analysis. Normalized gene expression for EMT-associated (*Twist1*, *Snai1*, *Vim*) and epithelial genes (*Cdh1*, *Ocln*). C, Analysis of circulating promoters of angiogenesis and metastasis. Serum *Vegf*, *Plgf*, osteopontin, *Scf* and *Cxcl12* were quantified by ELISA. Note that for all parameters assessed under A-C, DMOG consistently counteracted the ITPP effects, with no significant difference between samples treated with *saline plus vehicle*

and samples treated with *DMOG plus ITPP*. D, Kaplan-Meier survival analysis. Survival count (6-10 mice/group) starts with treatment end (day 15, dashed line).

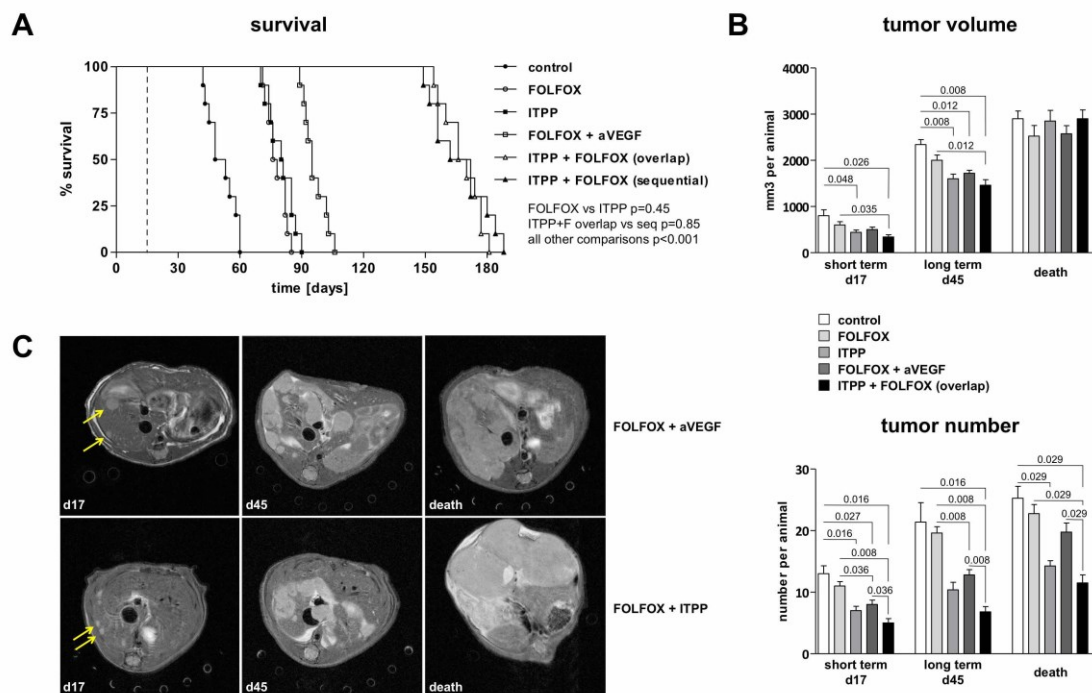
**Figure 5**



**Figure 5.** Long-term impact of ITTP on hypoxic response and tumor vasculature in the MC-38/B6 model. A, Long-term impact on the hypoxic response. Tumoral gene expression and serum quantifications are shown for control (-) and ITTP-treated mice (+) immediately (day 17) and one month (day 45) after treatment (see bottom for labels). Note the persistence of treatment effects for all parameters investigated. B, Long-term impact on tumor vasculature.

Immunohistochemistry for Cd31, Cd34, Notch1 and VE-cadherin in control and ITPP-treated tumors at day 45. Fading of background color was applied for better visualization of staining patterns. Please compare with Supplementary Fig. 6 to note the similar expression patterns immediately and one month after ITPP treatment.

**Figure 6**



**Figure 6.** Efficacy of ITPP treatment combined with chemotherapy versus standard-of-care treatment. To evaluate the efficacy of ITPP in potentiating standard chemotherapy against CRC liver metastases, tumor-bearing mice (the MC-38/B6 model) were treated with ITPP, FOLFOX, ITPP plus FOLFOX (overlapping or sequential), and FOLFOX plus Vegf antibody. A, Efficacy assessed by survival. Dashed line indicates treatment end. Ten mice/group were included. B, Efficacy assessed by tumor volume and number. MRI scans to define volume and number were performed immediately after treatment (day 17), four weeks after treatment (day 45) and at death. C, Representative MRI scans of mice treated with FOLFOX plus Vegf antibody or FOLFOX plus sequential ITPP.

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